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FULL-LENGTH ARTICLE

Manufacturing processes

Good Manufacturing Practice–compliant human induced pluripotent stem cells: from bench to putative clinical products

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ABSTRACT

Background aims: Few human induced pluripotent stem cell (hiPSC) lines are Good Manufacturing Practice (GMP)-compliant, limiting the clinical use of hiPSC-derived products. Here, we addressed this by establishing and validating an in-house platform to produce GMP-compliant hiPSCs that would be appropriate for producing both allogeneic and autologous hiPSC-derived products.

Methods: Our standard research protocol for hiPSCs production was adapted and translated into a GMP-compliant platform. In addition to the generation of GMP-compliant hiPSC, the platform entails the methodology for donor recruitment, consent and screening, donor material procurement, hiPSCs manufacture, in-process control, specific QC test validation, QC testing, product release, hiPSCs storage and stability testing. For platform validation, one test run and three production runs were performed. Highest-quality lines were selected to establish master cell banks (MCBs).

Results: Two MCBs were successfully released under GMP conditions. They demonstrated safety (sterility, negative mycoplasma, endotoxins <5.0 EU/mL and negative adventitious agents), cell identity (>75% of cells expressing markers of undifferentiated state, identical STR profile, normal karyotype in >20 metaphases), purity (negative residual vectors and no plasmid integration in the genome) and potency (expression of at least two of the three markers for each of the three germ layers). In addition, directed differentiation to somatoids (skeletal muscle precursors) and six potential clinical products from all three germ layers was achieved: pancreatic islets (endoderm), kidney organoids and cardiomyocytes (mesoderm), and keratinocytes, GABAergic interneurons and inner-ear organoids (ectoderm).

Conclusions: We successfully developed and validated a platform for generating GMP-compliant hiPSC lines. The two MCBs released were shown to differentiate into clinical products relevant for our own and other regenerative medicine interests.

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Introduction

Human induced pluripotent stem cells (hiPSCs) are now emerging as increasingly relevant regenerative medicine products. Among

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those with reported success are dopaminergic cells in Parkinson disease [1], retinal cells for macular degeneration [2], beta cells for the treatment of type I diabetes [3] and mesenchymal stromal cells in graft-versus-host disease [4]. The clinical application of hiPSCs requires that their production meets Good Manufacturing Practice (GMP) standards to ensure quality and safety of the product [5,6]. Although several protocols have been published for the production of GMP-compliant hiPSCs [7,8], the number of GMP-compliant hiPSC lines actually available remains limited, with the exception of a recently published large collection of a clinical-grade human leukocyte antigen (HLA) haplobank, matching approximately 40% of the Japanese population [9]. In addition to the HLA haplobank, the generation of universal hiPSC lines could provide a promising perspective for allogeneic cell therapy. Universal cell lines refer to cells that have been engineered to bypass the major histocompatibility complex mismatch. Achieving a universal hiPSC line would provide an “off-the-shelf” source for cell-based therapies reducing treatment wait time, eliminating donor search, reducing the need for immunosuppression and being a more cost-effective alternative to personalized autologous medicine [10].

As an academic hospital, the Leiden University Medical Center’s (LUMC) main interest is the development of innovative cell and gene therapy medicinal products and especially the application of hiPSC-derived products in patient care. Our aim was to establish a platform for in-house GMP-compliant hiPSCs production using the combined expertise of LUMC’s hiPSC hotel, specialized in developing research-grade hiPSC lines, and LUMC’s GMP facility (Centre of Cell and Gene Therapy [CCG]), with a proven track record in cell and gene therapy production [11–14]. This approach allowed the translation of a robust research protocol into a GMP production process. The translation involved, aside from using GMP-released raw materials and performing the manufacturing in a GMP-grade clean room, setting up a quality management system, training of personnel, generating a documentation system and design, validation and qualification of all necessary quality control (QC) tests for final product release. Aside from the generation of GMP-compliant hiPSC lines with demonstrated capacity for differentiation to multiple cell types relevant for cell therapy in regenerative medicine, our validated protocol could be used to produce patient-derived lines for personalized treatments. “Hospital exemption” for cell therapy in the Netherlands would create opportunities to test prospective clinical products.

Methods

Donor selection and screening of input material

Male donors (age 18–30 years) were recruited and signed consent forms to use their donated tissue for clinical research, pluripotent stem cell production, commercialization and whole-genome sequencing. For this project, peripheral blood was collected from each donor. A statement of “no objection” was obtained from the LUMC medical ethical committee for donor recruitment. Donors were screened according to EU directive 2006/17/EC and additional EU member state specific requirements. Dutch legislation on genetic modification considers episomal reprogramming as a genetic modification procedure requiring donor testing for the presence of pathogens (i.e., cytomegalovirus and Epstein–Barr virus) for which regulatory elements are present in the episomal vectors. Informed consent form, tests, medical examination, and inclusion decision are as described in [supplementary Table 1](#). All data were handled confidentially and in compliance with Good Documentation Practice in research. For anonymization, donors were allocated encoded

LUMC numbers, to which their tissue and data were linked; the key is safeguarded by the principal investigator.

Regulatory compliance of GMP manufacturing

Manufacturing was performed under European Union GMP regulation [5] following authorized standardized operating procedures generated as described in EudraLex Volume 4 Part IV - GMP requirements for Advanced Therapy Medicinal Products (ATMP) [6]. Clinical manufacturing was performed under aseptic conditions in a grade B clean room and a grade A laminar air flow cabinet.

Release of raw material for manufacture

Raw materials used were compendial or non-compendial and released for manufacture through in-house GMP risk analysis assessment. Episomal vectors, as described by Okita *et al.* [15]—pCXLE-hOCT3/4 (#27076), pCXLE-hSK (#27078) and pCXLE-hUL (#27080) (Addgene, Watertown, MA, USA)—were checked for integrity through restriction digest analysis and Sanger sequencing and subsequently produced in high quality ccc-classic grade (Plasmid Factory).

Isolation of peripheral blood mononuclear cells (PBMCs)

Peripheral blood samples (40 mL) were collected in 10-mL ethylenediaminetetraacetic acid tubes at the LUMC and transported to the in-house GMP facility (CCG) according to GMP-compliant standardized operating procedures. Samples were diluted with an equal volume of StemSpan-ACF Erythroid Expansion Medium (#09860; StemCell Technologies, Vancouver, Canada) followed by density gradient separation using Ficoll-Paque Premium (#17-5442-02; GE Healthcare, Chicago, IL, USA). PBMCs isolated from the Ficoll interphase were counted using the Nucleocounter NC-200 and $12\text{--}24 \times 10^6$ cells were used for erythroblast expansion. Leftover PBMCs were cryopreserved in CryoStor CS10 (#07952; StemCell Technologies) at a concentration of 5×10^6 cells/mL using a controlled-rate freezing system (Kryo 750; Planer, Sunbury-On-Thames, UK) for later-stage quality testing or, if necessary, a second production run. Cryopreserved PBMCs were stored in the vapor phase of a liquid nitrogen tank ($< -150^\circ\text{C}$).

Expansion of erythroblasts

An expanded erythroid progenitor cell population was used for reprogramming. PBMCs were seeded in 12–24 wells of a six-well cell culture plate at a density of 1×10^6 cells per well in StemSpan ACF supplemented with StemSpan Erythroid Expansion Supplement 100× (EES; #02692; StemCell Technologies). Cells were cultured in a 37°C incubator with 5% CO₂ and the culture medium refreshed daily. On day 2, cells in suspension were transferred to a new well to remove any adherent cells. Erythroblasts were harvested after 7–9 days when the cells exhibited visual signs of expansion.

Reprogramming of erythroblasts and colony selection

Erythroblast cell number and viability was assessed using a Bürker chamber in a 1:1 Trypan Blue (#15250061; Gibco) dilution. For reprogramming, two to four independent electroporations were performed per donor, using $0.5\text{--}1 \times 10^6$ live cells. Cell pellets were resuspended in P3-buffer (Primary Cell 4D-Nucleofactor Kit, #V4 × 9-3012; Lonza, Basel, Switzerland) containing 1 µg of each episomal vector. Electroporation was performed in a Nucleocuvette Vessel with 4D-Nucleofactor (Lonza) program EO-115. Subsequently, cells were resuspended in 6 mL of prewarmed (37°C) ACF supplemented with EES per cuvette and divided over six wells of a 12-well plate coated with 0.5 µg/cm² Laminin 521 CTG (#CT521; Bio Lamina

Table 1

Quality control tests performed during GMP-grade hiPSCs manufacture, materials tested and established acceptance criteria.

	Test name	Test material	Accepted outcome	IPM	IPC	MCB Release
Safety	Sterility, bacterial infection	Ficoll fraction	NEG	✓	✓	✓
		During the whole process on indication Supernatant at harvest P9/P10, MCB				
Identity/purity/impurity/	Mycoplasma	Ficoll fraction	NEG		✓	✓
		Supernatant at P3 and P9/P10 Cells and supernatant at harvest MCB				
	Endotoxin	Supernatant at harvest MCB	<5.0 EU/mL			✓
		Cells at harvest MCB	NEG			✓
	Cell morphology	Visual and imaging during cell handling	>90% of cells typical hiPSC morphology		✓	✓
		Colony morphology	Visual and imaging during cell handling	>90% of colonies typical shape	✓	
	Viability	Cells at harvest P9/P10, MCB	>70%			✓
		Residual vector	Cells at harvest at P7/8 (P9/P10), MCB	NEG		✓
	Markers of undifferentiated state	Cells at harvest P9/P10, MCB	>75% positive for SSEA4, Tra-1-60 and OCT3/4			✓
		STR profiling	Cells at harvest P9/P10, MCB	Identical to starting material		✓
	Karyotyping	Cells at harvest P9/P10, MCB	Normal (diploid) ≥ 20 metaphases			✓
		Whole-genome sequencing	Cells at harvest P9/P10	No (partial) plasmid integration		✓
Whole-exome sequencing*	Cells at harvest MCB, P15, P26	No ACMG class 4/5 variants in Cosmic genes			✓	
	KaryoStat*	Cells at harvest MCB, P26	No chromosomal aberrations found			✓
Potency	Trilineage differentiation	Cells of P9/P10, MCB	At least 2 of 3 markers positive for each germ layer		✓	✓

GMP, Good Manufacturing Practice; hiPSCs, human induced pluripotent stem cell; IPM, in-process monitoring; IPC, in-process control; MCB Release, Master Cell Bank release.

* For information only.

[Sundbyberg, Sweden]; 12–24 wells in total). On day 2 after seeding, 1 mL of ACF plus EES was added to each well. On day 3 and day 5 after seeding, 1 mL of mTeSR Plus medium (#100-0276; StemCell Technologies) was added. From day 7 onwards, cells were refreshed with 1 mL of mTeSR Plus medium every second day until hiPSC colonies appeared. For picking, each colony was “cut” into 6–12 pieces and transferred to a Laminin-coated well of a 12-well plate. Picked colonies, labeled as passage 1, were considered clonal and treated as independent lines. All lines were coded as follows: LUMC-GMP-iPSC_DxPxLx (Dx: donor number, Px: passage number and Lx: line number).

Maintenance of hiPSC lines

Cells were passaged (every 6–7 days based on colony morphology, size, and confluency) as 300–500 aggregates of 50–200 μm on laminin (P2) or vitronectin (0.9 $\mu\text{g}/\text{cm}^2$) (#A27940; Gibco, Waltham, MA, USA)-coated wells of a six-well plate (P3 onwards) containing 2 mL of mTeSR Plus medium by incubating them with Versene CTS (#A4239101; Gibco) for 4 min at room temperature (RT). Medium was refreshed every 1–3 days.

Cryopreservation of hiPSC lines

hiPSCs were cryopreserved at passages 3, 5, 7, 9, 10 and 12 for quality testing and banking. Cell aggregates equivalent to half a well of a six-well plate (P3-P7 and P12) or the equivalent of one well of a six-well plate (P9/P10) were frozen in 1 mL of CryoStor. Freezing was performed in a controlled-rate freezing system (Kryo 750; Planer). Cryopreserved hiPSCs were stored in the vapor phase of liquid nitrogen ($< -150^\circ\text{C}$).

Master cell bank (MCB) generation

Three lines of one donor at passage P10 were selected for generation of the MCB. Five vials cryopreserved at P10 were plated onto 10 wells of six-well plates coated with vitronectin in mTeSR Plus medium supplemented with 10 $\mu\text{L}/\text{mL}$ RevitaCell (100 \times stock, #A4238401; Gibco). Medium was refreshed the next day to avoid prolonged exposure of the cells to RevitaCell. After 6–7 days, cells were passaged to eight vitronectin (0.9 $\mu\text{g}/\text{cm}^2$)-coated T175 flasks in 40 mL of mTeSR Plus medium per flask for scale-up. Cells were maintained as described previously and harvested at P12 using

18 mL of Versene CTS per flask for 4 min at RT. Cell aggregates from seven flasks were pooled and cryopreserved in CryoStor CS10 in 120 vials and the last flask was used for QC testing.

QC testing

Sample preparation for QC tests

To assess sterility, mycoplasma and endotoxin levels, the culture supernatant from all wells or flasks was collected and pooled. Matrix verification was performed to ensure the validity of the assays. For cell viability, the undifferentiated state, trilineage differentiation, residual vector analysis, STR-profiling, whole-genome/exome sequencing and adventitious agents testing, hiPSCs were dissociated into single cells by incubation with 18 mL of Versene CTS at 37°C for 10 min. For karyotyping, a small sample was taken of cell aggregates from the pooled pellet obtained after MCB harvest. Table 1 shows QC tests performed, material used and acceptance criteria.

Cell and colony morphology

The cells were examined under a Leica M80 stereomicroscope at every step of the manufacturing process to confirm typical morphology and to monitor the degree of spontaneous differentiation. Before passaging, colony and cell morphology were checked and imaged using an inverted microscope (Zeiss Primovert/Axiocam-105c).

Cell count and viability

Cell numbers and viability after harvest was assessed using the Nucleocounter NC-200 according to European Pharmacopoeia (Ph. Eur.) 2.7.29 Nucleated cell count and viability.

Sterility testing

Sterility testing was performed at the LUMC's accredited microbiology laboratory according to Ph.Eur. 2.6.27, Microbial Examination of cell-based Preparations (aerobic and anaerobic microbial contamination) procedures using BacTec flasks (#442023 and #442022; BD, Franklin Lakes, NJ, USA).

Mycoplasma testing

Mycoplasma tests were performed at the LUMC's accredited microbiology lab with PCR in compliance with Ph.Eur. 2.6.7., *Mycoplasmas*.

Endotoxin testing

Endotoxin testing was performed at the LUMC's accredited Clinical Pharmaceutical Lab using the LAL test and complies with Ph.Eur. 2.6.14, *Bacterial Endotoxins*.

Adventitious agents testing (AAT)

AAT was performed using the Twist Comprehensive Viral Research Panel. The assay was performed by and validated for sensitivity and reproducibility by GenomeScan BV (Leiden, Netherlands). The validation process was supervised and reviewed by the QC ATPM team of the LUMC GMP facility (CCG).

Residual episomal vector analysis

gDNA was extracted from a maximum of 5×10^6 hiPSCs at the indicated passage numbers using a DNeasy Blood & Tissue Kit (#69504; QIAGEN, Hilden, Germany) according to the manufacturer's protocol. The presence of residual reprogramming vectors was tested with a quantitative polymerase chain reaction (qPCR)-based assay using primers annealing to the gene encoding for *EBNA-1* present in each of the reprogramming vectors and primers annealing to hexokinase 2 (*HK2*) as a reference for DNA input. EBNA-F-primer (5'-CAAGGAGGTTCCAACCCGAA-3'), EBNA-R-primer (5'-GACCCAAGTTCCTTC GTCGG-3'), HK2-F-primer (5'-GCCGACTTTGTATTGCTG-3') and HK2-R-primer (5'-TATTGTAG-CACGGCCGAAA-3'). qPCR was performed using PowerTrack SYBR Green Master Mix (#A46109; Thermo Fisher Scientific, Waltham, MA, USA), reverse and forward primers (final concentration 0.25 μ mol/L), and 120 ng of genomic DNA on the Quant Studio 5 (Thermo Fisher Scientific) for 35 cycles. A negative PCR signal (no residual vector detected) was set to a CT value of 35. The assay was validated and qualified in-house.

Analysis of markers of the undifferentiated state

Dissociated cells were fixed and permeabilized using BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit (#554714; BD Biosciences) according to the manufacturer's recommendations. Permeabilized cells (2×10^5 cells per fluorescence-activated cell sorting [FACS] tube) were incubated at 4°C for 30 min with the following antibodies at the respective dilutions: TRA-1-60 BV510 at 1:100 (#563188; BD Biosciences), SSEA-4 FITC at 1:3200 (#560126; BD Biosciences) and OCT3/4 PerCP-Cy5.5 at 1:50 (#560794; BD Biosciences). Cells were washed with Perm/Wash buffer and FACS buffer (PBS/0.5% ethylenediaminetetraacetic acid), resuspended in 150 μ L of FACS buffer and subjected to spectral flow cytometry using the Cytex Aurora (Cytex Biosciences, Fremont, CA, USA). The assay was validated and qualified in house.

Short tandem repeats (STR) profiling

STR profiling of primary PBMC and hiPSCs was performed by the accredited department of Clinical Genetics of the LUMC. The STR profile of the hiPSCs was compared with the input PBMC to ensure authenticity of the hiPSC lines.

Karyotype analysis by G-banding and KaryoStat

Undifferentiated hiPSC cultures of 5–6 days were treated with KaryoMAX Colcemid solution (#15212012; Thermo Fisher Scientific) for 1 h at 37°C to arrest cells in metaphase and processed further by the LUMC's accredited Diagnostic Genome Analysis laboratory (Leiden, Netherlands) for G-banding. At least 20 metaphases were examined for cytogenetic aberrations. Karyotypes were described according to the International System for Human Cytogenetic Nomenclature. Karyotype analysis was verified using the KaryoStat+ assay performed by Thermo Fisher Scientific on hiPSCs harvested at P10 and \geq P26.

Whole-genome sequencing (WGS) and whole-exome sequencing (WES)

To assess possible integration of reprogramming vectors, WGS was performed using the NovaSeq6000 platform (Illumina, San Diego, CA, USA) at P9/10. WES was performed using the NovaSeq6000 platform and the Agilent SureSelectXT human all exon v7 capture library (Illumina) on MCB samples at P12 and P26. Both assays were outsourced to the accredited company GenomeScan.

Downstream analysis of WGS and WES data

For single nucleotide polymorphism/indel calling, and structural variant analyses, the Illumina DRAGEN output was used on donor-derived PBMCs and hiPSC lines. Variants in the exon coding region were double checked using WES data generated from hiPSC lines at P12 (MCB), and P26. The workflow, based on GATK-compliant algorithms, entails quality control and a read preprocessing stage in which adapter sequences, low-quality regions (phred < 20) and poly G trimming was performed. Reads shorter than 20 bp were discarded. Subsequently, reads were mapped with a short read aligner to the indexed reference sequence (GRCh37/hg19). The resulting alignment (BAM) files were sorted on coordinate and indexed for downstream analysis. Copy number variations (CNV), in VCF file format, were filtered on a minimal length of 100 000 bp, a QUAL threshold of 25 and annotated using AnnotSV 2.2. Short variant calling was performed with the DRAGEN variant caller. Filtering was applied on the resulting VCF file on known COSMIC cancer gene census Tier 1/2 (version 96) to screen over 700 tumor suppressor- and oncogenes for the presence of pathogenic single nucleotide polymorphisms (COSMIC Census, <https://cancer.sanger.ac.uk/census>). Any *de novo* variants introduced were screened by reporting the variants only observed in the hiPSC lines and not in the donor PBMC reference sample. Hard-filtering of short variants was performed according to filtering criteria recommended by the Broad Institute (<https://gatk.broadinstitute.org/hc/en-us/articles/360035890471-Hard-filtering-germline-short-variants>) as follows: QD < 2; MQ < 40; MQRankSum < -12.5; ReadPosRankSum < -8.0; SOR > 4. Additional applied filters are: DP < 6; GQ_patient score < 7; MAX_AF > 0.05. For variants called against COSMIC, the following workflow was followed: Missense variants in protein coding regions, and variants in the 3' and 5' UTR and splice-regions were manually checked using integrative genomics viewer (IGV) [16]. Variant reference numbers were checked using the online variant annotation program Franklin by Genoox: <https://franklin.genoox.com>. Franklin scores are aggregated from public databases and are based on the American College of Medical Genetics and Genomics (ACMG) Guidelines [17].

Analysis of differentiation capacity

Trilineage differentiation was performed using the StemDiff Trilineage Differentiation Kit (#05230; StemCell Technologies) according to the manufacturer's recommendation. Subsequently, cells were fixed with 2% paraformaldehyde (PFA; #16005; Sigma-Aldrich, St. Louis, MO, USA). Differentiation capacity was confirmed by immunocytochemistry (ICC) using lineage-associated markers. Fixed coverslips were washed twice with phosphate-buffered saline (PBS) before incubation with Triton blocking solution (4% normal swine serum; #014-000-121; Jackson, ImmunoResearch Laboratories, West Grove, PA, USA) and 0.1% Triton X-100 (#93443; Sigma-Aldrich) in PBS) for 1 h at RT. Coverslips were washed with PBS and incubated overnight at 4°C with custom-made pre-labeled antibodies from Cell Signaling Technology in 4% normal swine serum/PBS with 1:1000 DAPI (#62248; Thermo Fisher Scientific) as follows: for ectoderm FAPB7 (D8N3N) at 1:100 (Alexa-555), PAX6 (D3A9V) at 1:200 (Alexa-647) and Nestin (10C2) at 1:200 (Alexa-488); for endoderm FOXA2 (D56D6) at 1:500 (Alexa-555), GATA4 (D3A3M) at 1:200 (Alexa-647) and EOMES (D8D1R) at 1:100 (Alexa-488) and for mesoderm CDX2

(D11D10) at 1:500 (Alexa-555), Vimentin (D21H3) at 1:400 (Alexa-647) and Brachyury (D2Z3J) at 1:200 (Alexa-488). Before mounting, coverslips were washed three times with 0.05% Tween-20 (#822184; Merck, Rahway, NJ, USA)/PBS for 10 min in the dark. Analysis was performed on the LSM900 Airyscan confocal microscope (Zeiss, Jena, Germany). The differentiation kit and trilineage differentiation analysis were validated and qualified in-house.

Stability testing

To monitor the effect of long-term storage, a stability study protocol was established in which the post-thaw sterility, viability, identity, and potency of the cryopreserved product is and will be assessed at 6 months, 1 year, 2 years, 5 years and 10 years.

Differentiation to prospective clinical products

SC-islets (hiPSC-derived pancreatic islets)

hiPSC lines were switched to expansion culture on vitronectin-coated plates (#A14700; Thermo Fisher Scientific) in Essential 8 medium (#A1517001; Thermo Fisher Scientific) and passaged using Versene (#15040-033; Gibco). Differentiation towards pancreatic islets was conducted using a fully clinically-compliant protocol in three-dimensional suspension. The seven-stage protocol (30 days) was adapted from previously reported protocols. To summarize, hiPSC cultures were dissociated to single cells using Accutase (#07920; StemCell Technologies) and seeded at a density of 0.5×10^6 cells/mL in a disposable bioreactor (ABLE Biott; ABBWVS03A-6). After 48 h, to allow cell cluster formation, the medium was changed to induce definitive endoderm (stage 1, 3 days), then primitive gut tube (stages 2, 3 days), posterior foregut (stage 3, 2 days), pancreatic endoderm (stage 4, 4 days), endocrine precursor (stage 5, 4 days), hormone expressing cells (stage 6, 8 days) and finally, maturing cells (stage 7, 7 days) (see supplementary Table 4 for media used). Cells were analyzed for relative gene expression of beta cell markers INS, PDX1, NKX6.1, MAFA, staining of SC-islet clusters with dithizone, and the expression of beta-cells and alpha-cell hormones, C-peptide and glucagon, by FACS and ICC.

Kidney organoids

hiPSCs were switched to Essential 8 medium (A1517001; Life Technologies, Carlsbad, CA, USA) and differentiated using previously described protocols [18,19]. To summarize, 15,000 hiPSC/cm² were incubated for 4 days in 8 μ mol/L CHIR99021 (#4423; Tocris, Bristol, UK), in STEMdiff APEL2 medium (# 05275; StemCell Technologies). On day 4, growth factors were changed to 200 ng/mL rhFGF9 (#273-F9; R&D Systems, Minneapolis, MN, USA) and 1 μ g/mL heparin (#H3149; Sigma-Aldrich). On day 7, cells were transferred as small clumps to 3D culture on Transwell 0.4- μ m pore polyester membranes (#3450; Corning, Corning, NY, USA) and maintained for another 18 days. Organoid quality was assessed on day 25 with brightfield microscopy and ICC. Organoids were fixed in 2% PFA and stored in PBS. Whole mount tissues were stained for specific kidney markers (CD31, NPHS1, NPHS2, CUBN, LTL and ECAD) and examined using the White Light Laser Confocal Microscope TCS SP8 (Leica, Wetzlar, Germany).

Cardiomyocytes

hiPSCs were differentiated into cardiomyocytes and analyzed by FACS and ICC as described previously by van den Brink *et al.* [20]. To summarize, on day -1, hiPSCs were dissociated to single cells and plated on laminin-coated 12-well plates in StemFlex media supplemented with 10 μ mol/L ROCKi at $2.0\text{--}2.5 \times 10^5$ cells/well. On day 0 the medium was replaced with mBEL with CHIR99021 (5 μ mol/L). On day 2, cells were refreshed with mBEL supplemented with XAV939 (5 μ mol/L) and IWP-L6 (0.25 μ mol/L). From day 4 onwards,

cells were refreshed every 2–3 days with mBEL containing iTS-X until harvest on day 17. Harvested cells were used for FACS analysis of cardiac troponin T expression or plated on coverslips and fixed on day 24 for α -actinin and cardiac troponin T ICC analysis.

Keratinocytes

hiPSCs were differentiated to keratinocytes as previously described by Ruiz-Torres *et al.* [21] with minor modifications. To summarize, hiPSC were seeded on Matrigel hESC-qualified matrix (#354277; Corning)-coated 6 well plates at approximately 1 clump/cm² in StemFlex medium (#A3349401; Day -1; Thermo Fisher Scientific). On day 0 and 2, the medium was replaced by Defined Keratinocyte SFM (#10744019; Thermo Fisher Scientific) containing 1 mmol/L all-trans retinoic acid (#R2625; Sigma-Aldrich) and 25 ng/ μ L BMP4 (#314-BP; R&D). Cultures were subsequently maintained in Epithelial Proliferation Medium (#CnT-07; CELLnTEC) supplemented with 10 μ mol/L Y27632 from day 10 onwards. At day 20, cells were analyzed by FACS for the expression of α 6-integrin (#GoH3; BioLegend, San Diego, CA, USA) and β 4-integrin (#439-9B; BioLegend).

Neural lineage

hiPSCs were first differentiated for 16 days into medial ganglionic eminence (MGE) progenitors and then matured into GABAergic interneurons for 42 days. A detailed description of the differentiation process can be seen on the supplementary Table 5. The gene expression profile of the cells differentiated toward neural lineage were analyzed after day 16 and day 42 of the differentiation process through qRT-PCR. Analyzed genes were PAX6, FOXG1, NKX2.1, LHX6, ISL1, SST, PVALB and GAD67. For normalization the housekeeping genes ACTB and glyceraldehyde 3-phosphate dehydrogenase were used. The fold change of the differentiated samples was calculated relatively to undifferentiated embryonic stem cell (hESC) RC17 reference. Cells were fixed with 4% PFA at day 16 and day 42 for ICC analysis. Antibodies used for MGE progenitors (day 16) were NKX2.1 (1:200; #343M-96; Sigma-Aldrich), ISL1 (1:40; #39.45D; DSHB, Iowa City, IA, USA) and PAX6 (1:1000; #AMAb91372; Sigma-Merck). For GABAergic interneurons (day 42) GABA (1:1000; #A0310; Sigma-Aldrich), SST (1:200; #T-4103; BMA Biomedicals, Basel, Switzerland) and MAP2 (1:1000; #M1406; Sigma-Aldrich) were used. All ICC samples were co-stained with DAPI (1:500; #D3571; Invitrogen, Waltham, MA, USA).

Inner-ear organoids

hiPSC lines were differentiated toward inner ear organoids following the protocol described by van der Valk *et al.* [22,23], with minor alterations. In brief, hiPSC colonies were seeded as a single-cell suspension on 96-well U-bottom plates with a super-low cell attachment surface (#174925; Thermo Fisher Scientific). In total, 2500 cells were seeded per well in E8 medium containing chroman 1 (#HY-15392; MedChem Express, Monmouth Junction, NJ, USA), emricasan (#HY-10396; MedChem Express), polyamine (#P8483; Sigma-Aldrich) and trans-ISRIB (#5284; Tocris) (CEPT) as described by Chen *et al.* [24]. Following a 48-h incubation, cranial neural crest induction was initiated at day 0 of differentiation. On day 8 of differentiation otic placode formation was induced. The aggregates were transferred to organoid maturation medium on day 12 until day 75. Formalin-fixed paraffin-embedded organoids were analyzed on day 75 by ICC using antibodies directed against MYO7A (1:100; Proteus, #25-6790) and TUBB3 (1:100; BioLegend, #801202)

Somitoids

Somitoids were generated according to the protocol described by Sanki-Matsumiya, *et al.* [25,26] with minor modifications. To summarize, hiPSCs were dissociated with Versene to single-cells and washed in N2B27 medium supplemented with CEPT cocktail [24]. hiPSCs were resuspended in somitoid differentiation medium supplemented

with CEPT cocktail and 350 cells were seeded in Nunclon Sphera 96-well microplate (#174925; Thermo Fisher Scientific). The plate was centrifuged for 2 min at 150g and then incubated overnight at 37°C and 5% CO₂. After 24 hours 150 µL of somitoid induction medium without CEPT cocktail was added to each well. 48 and 72 h after aggregation, medium was replaced with N2B27 medium. At 96 h after aggregation, about 10 elongated somitoids were collected and embedded into 10% Cultrex RGF Basement Membrane Extract, Type 2 (#3533-010-02; R&D Systems) and incubated at 37°C and 5% CO₂. 48h after embedding, the somitoids were collected for HCR RNA-FISH analysis and ICC.

Results and Discussion

Critical steps involving donor selection, screening of input material, translation of the research protocol to a GMP-compliant production process and QC testing are described herein. For platform validation, a minimum of three production runs was required. Hence, three donors were recruited for GMP production up to cryopreservation and QC testing of the seed lot. GMP manufactured seed lots passing all controls, including in-process monitoring, in-process control and QC tests, were qualified person (QP)-released. Production of three MCBs was required including their full QC release testing. MCBs passing all QC release testing were GMP released.

Donor selection and screening of input material

Male donors were chosen to avoid possible erosion of X chromosome inactivation described for hiPSC lines from female donors [27]. Tissue from three donors consenting to research use was used under standard laboratory conditions to translate the research reprogramming protocol to a GMP-compliant process. Ten lines were first generated from each donor and used for validating hiPSC-specific release tests. The next run was a test in the GMP facility, using PBMC from a bone marrow donor who consented to research use of surplus donated material. For the three GMP production runs, donors were recruited from November 2021 through March 2022. These donors consented to the use of their material for clinical research, stem cell production, commercialization and whole-genome sequencing. All donors passed the selection criteria and medical examination outlined in supplementary Table 1; these tests were performed on the day of blood collection. To exclude potentially missed infectious agents during the initial material collection, donors consented to a second blood collection 6 months after their first donation for repeated testing. Medical examinations were performed by an internist. Donated material was released for ATMP production by a qualified hematologist and a QC ATMP officer.

Translation of the research protocol to a GMP protocol

PBMCs were used as input material for erythroblast expansion and subsequent reprogramming. The in-house reprogramming protocol for generating research grade hiPSCs underwent a number of changes to translate the process to GMP. First, we replaced research-grade erythroid expansion medium containing bovine serum albumin with an animal component-free erythroid expansion medium. Second, instead of pCXLE-OCT3/4-shRNA-p53, which contains an shRNA against p53, we used pCXLE-Oct3/4 plasmid without the p53 knockdown. The rationale for omitting p53 knockdown was related to potential safety issues in patients, where an intact p53 (tumor suppressor) pathway would be advisable. ReproTeSR was replaced by mTeSR-Plus as reprogramming medium because its composition is known. All media were free of antibiotics. In addition, (undefined, animal-derived) Matrigel was replaced by laminin 521 CTG (first three passages) and vitronectin-CTS (later passages). During reprogramming laminin supported superior colony outgrowth and up to

40% more colonies formed. However, on vitronectin, hiPSC colony morphology was more homogenous which facilitated elimination of spontaneous differentiation and better culture maintenance at later passages. These modifications to the research protocol did reduce erythroblast expansion and reprogramming efficiency. Compared with Okita and Yoshida, reprogramming using our GMP protocol was 100–1000 times less efficient [9,15]. We therefore scaled up to 24 × 10⁶ PBMCs for erythroblast expansion and performing multiple electroporations per donor in parallel to increase the number of colonies obtained. We noted that erythroblast expansion rate and reprogramming efficiency were highly donor dependent, varying from 3 to 300 colonies per donor. Details of erythroblast expansion and reprogramming efficiency are shown in [supplementary Table 2](#).

GMP-compliant hiPSCs production

Individual colonies were picked between day 14 and 24 after electroporation. To avoid cross-contamination of colonies, we only selected those that were isolated from other colonies and picked only one colony per well. One colony was placed in one well of an independent 12-well plate, designated passage 1 and assigned a unique line number for identification (DxPxLx). Each of these lines was considered an individual product, meaning that from passage 1 onwards different lines were never handled simultaneously. From passage 2 onwards, colonies were maintained in six-well plates and passed as aggregates once every 6–7 days as soon as they had centers that were dense and phase-bright compared to their edges. Every second passage, several wells of each line were cryopreserved. Selection of lines during the production process was based on visual monitoring of spontaneous differentiation, colony morphology and episomal plasmid retention.

An overview of all the GMP-hiPSC lines generated and their QC data is shown in supplementary Table 3. “Pass” or “fail” for acceptance criteria and reasons for exclusion are indicated. Exclusion during the GMP production process was primarily based either on failure to expand after colony picking or more than average levels of spontaneous differentiation. After 8–9 passages, the selected lines appeared to be stable, displaying less than 10% spontaneous differentiation and expected colony morphology. In process control testing entailed checking for residual episomal vector, which was performed on passage 8 for donors D1, D2 and D3 and passage 7 for donor D4. The limit of detection for qPCR on the episomal vector was agreed with the Dutch Office of Genetically Modified Organisms (B-GMO) and set at 1 copy per 1000 cells. qPCR, using primers annealing to the *EBNA-1* gene, was performed on 16 clones, of which 11 had no detectable episomal vector at passage 7–8 (i.e., more than 20 EBNA-positive cells per input of 20 000 cells equaling the set limit of detection) after 35 cycles. In four of the 16 lines tested (D4L08, D4L14, D4L16 and D4L18) residual episomal vector was detectable with CT values between 22 and 33 (see [Figure 1](#)); these were excluded and removed from the manufacturing process. Finally, clone D4L01 displayed inconclusive results (CT value of 34) at passage 7. It was kept in culture and tested negative at passage 10.

Rejection of lines due to residual episomal vectors occurred in 25% of cases, which is in line with previous reports [9,28–31]. Our results suggest that screening between passages 8 and 10 is sufficient to exclude lines with episomal vector retention. Based on the results of the residual vector analysis shown in [Figure 1](#), lines D1L03, D1L04, D1L06, D2L01, D2L02, D2L03, D3L13, D3L14, D3L19, D4L01, D4L10 and D4L17 were selected. These 12 lines were scaled up to 24 wells, cryopreserved as pre-seed lots and subjected to further QC testing, as outlined in [Table 1](#).

To exclude partial integration of the reprogramming vector, which could be missed with qPCR detecting only a part of the plasmid backbone, WGS analysis was performed at P9/P10 with a minimum sequence coverage of 50×. Reads were mapped to the pCXLE-

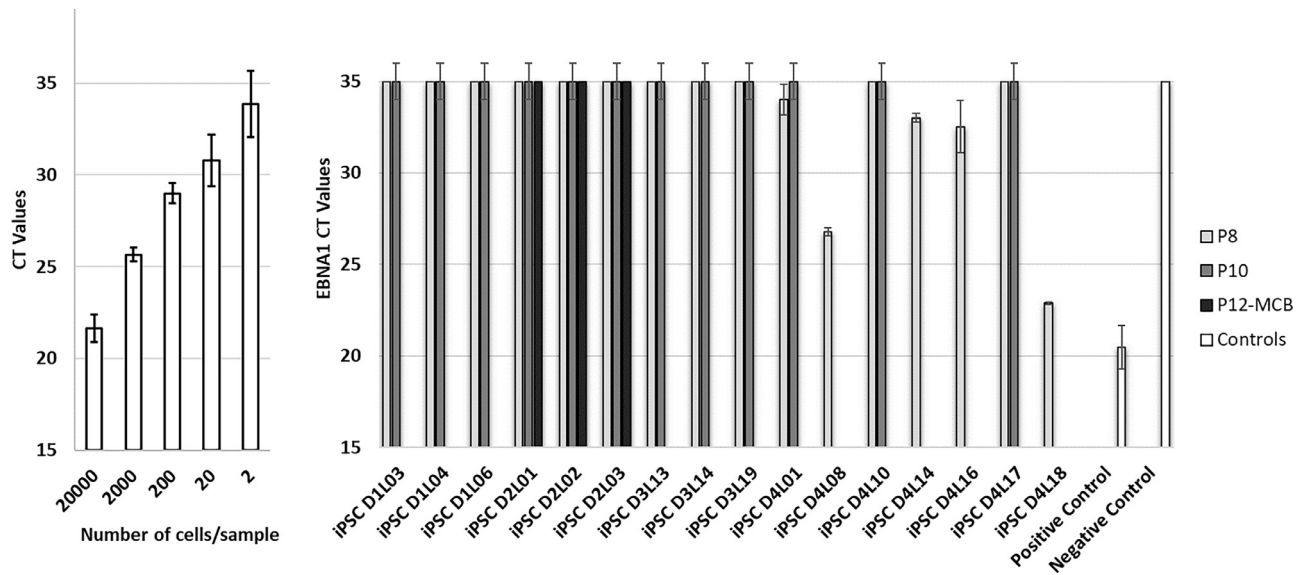


Figure 1. Residual episomal vector qPCR: Screening for the presence of residual reprogramming vectors at P7-8, P9-10 and MCB. Left panel demonstrates the correlation between CT values and number of hiPSCs used as input sample. Right panel displays the average EBNA1 CT values for each line ($n = 3$) and for the controls ($n = 18$).

backbone reference. Mapped reads were checked for biological significance to exclude mapping artifacts. The selected lines of D2 and D4 did not show genomic DNA vector integration, confirming the results of qPCR screening. Samples from D1 and D3 were not included in this analysis (see to follow).

Accepted outcome parameters for QC tests were either established (e.g., mycoplasma, sterility and endotoxin), based on acceptance criteria for other GMP production processes in the LUMC (e.g., viability and karyotyping), or formulated at the start of assay validation (hiPSC-specific release tests). Recommendations for hiPSC-specific release criteria have been published and guided us in setting acceptance criteria for the undifferentiated state of hiPSCs and their differentiation potential [32,33]. Markers associated with the undifferentiated state and self-renewal (OCT3/4, SSEA-4 and TRA-1-60) were analyzed by FACS. Acceptance criteria were set as the presence of individual markers in at least 75% of the cells. The selected 12 lines displayed all three markers in at least 85% of the cells (example shown in Figure 2B and summarized in supplementary Table 3). To assess the differentiation potential *in vitro*, three markers representative of each of the three germ layers were analyzed by ICC. The release criterion for this was set at a minimum of two positive markers per germ layer. All 12 selected lines, three from each of the four donors, demonstrated functional pluripotency (example shown in Figure 2A) and passed additional QC release tests performed at P9-P10 (see supplementary Table 3).

Beside reprogramming vector retention, defects in genetic integrity are also reasons to reject hiPSC lines for clinical applications. We therefore subjected donor PBMCs to genetic analysis by WGS. D1 was excluded from this analysis because the consent obtained for research applications did not contain consent for WGS. Variant calling of donor PBMC against COSMIC gave the following results: a total of 104 variants were found in PBMCs of D2 of which 46 were ACMG class 1, (benign) 22 class 2 (likely benign), and 36 class 3 (variant of unknown significance, VUS). Importantly none were class 4 (likely pathogenic) or class 5 (pathogenic). D4 displayed a total of 47 variants made up of 14 class 1, (benign) 4 class 2 (likely benign) and 29 class 3 (VUS) without any ACMG class 4 or class 5 variants. By contrast, genomic analysis of D3 PBMCs revealed a germline class 5

pathogenic deletion in one of the COSMIC oncogenes. Hence, pre-seed lines of D3 did not pass QP release and were not subjected to WGS. The combined results of pre-seed lot QC testing culminated in QP release of 6 pre-seed lots: three from D2 (D2P10L01, D2P10L02, D2P10L03) and three from D4 (D4P9L01, D4P9L10, D4P9L17).

Selection of lines and production of MCB

The three pre-seed lots of D2 were selected for validation of MCB production based on donor characteristics. Blood type incompatibility can cause acute transplant rejection [34]. Therefore, donor D2 with AB0 type 0+ was preferred to D4 with AB0 type B+. Scale-up to allow cryopreservation of 120 MCB vials was achieved by seeding 5 pre-seed lot vials in 10 wells of 6-well plates. MCB production D2MCBL01B was successful after the first attempt (D2MCBL01) failed due to infection. Next to all QC tests performed on the pre-seed lots, additional QC tests for endotoxin, AAT and WES/WGS were performed on the MCBs. For AAT, a next-generation sequencing-based method was used. We used the highly sensitive comprehensive viral research panel developed by Twist Biosciences, capable of screening for >3000 different viruses, including >15 000 viral strains. This method is able to detect viruses that are missed using conventional AAT much faster, does not require animal testing, and is more cost-efficient compared to conventional AAT. While its suitability is still under debate, reports from workshops organized with regulatory agencies predict authorization of NGS-based AAT in the near future [35,36]. No viral contamination was observed in the MCBs tested.

The three lines passed all tests except for D2LMCBL02, which, unexpectedly, displayed an abnormal karyotype (DEL Chr11:q22.1-23.3) in all 20 metaphases analyzed at P12, whereas at P10 this line scored as normal. Since it is unlikely that 2 passages were sufficient for cells with the karyotype abnormality to overtake the culture, the P10 karyogram was re-assessed. This revealed that the karyotype abnormality was already present in P10 and due to its size and position difficult to score and hence missed in the first screen. This prompted additional chromosomal integrity testing using KaryoStat (resolution 1–2 Mb), complementing the G-banding (resolution 5–10 Mb) results by providing accurate genotyping and detection of submicroscopic aberrations. The deletion in chromosome 11, which

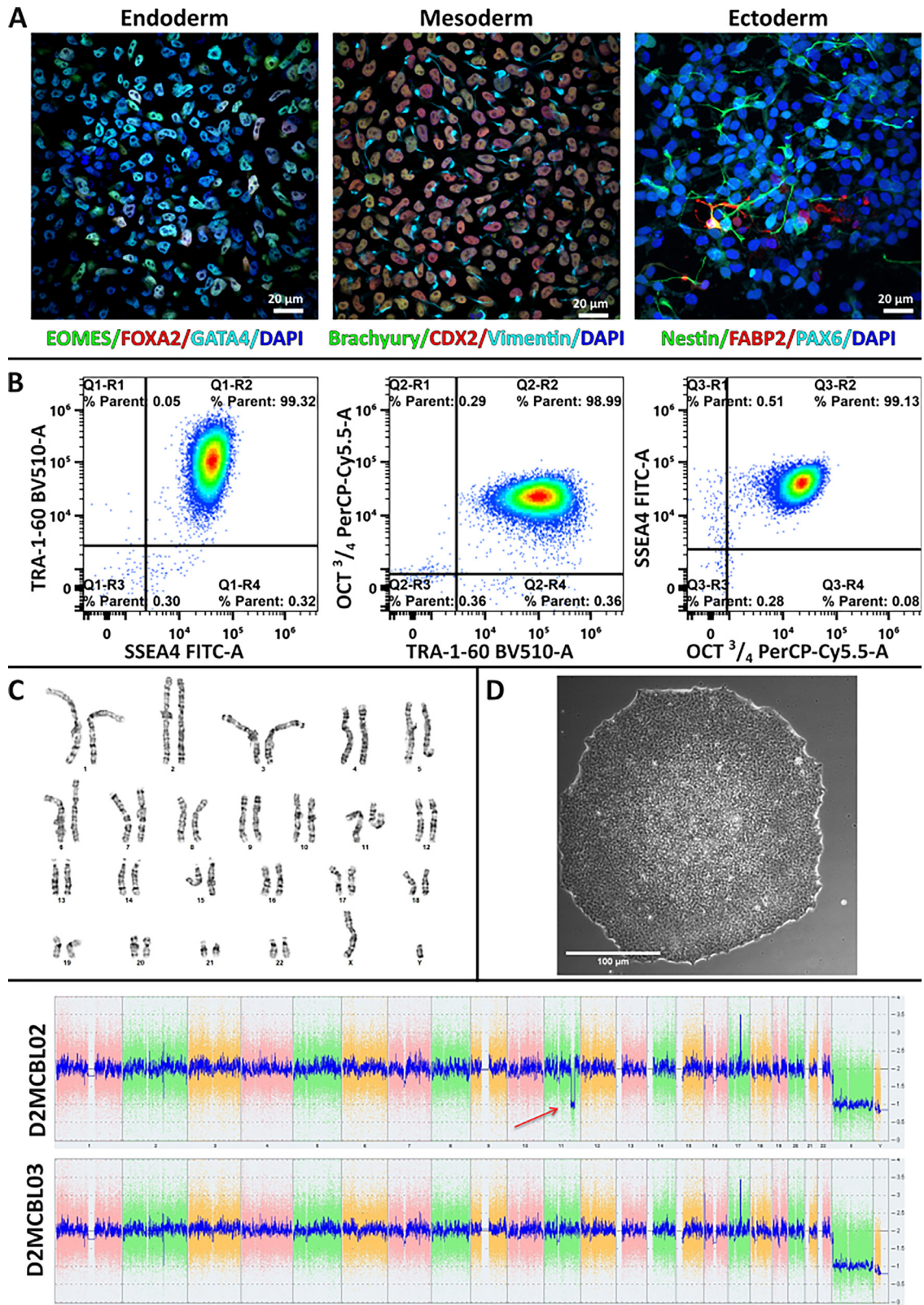


Figure 2. Representative images of QC tests performed on D2MCBL03: (A) Confocal microscopy of TD. (B) Markers of undifferentiated state. (C) Karyogram. (D) Colony morphology on day of harvest (scale bar 20 μ m). (E) KaryoStat analysis: Raw signal for each individual chromosome probe (pink, green and yellow), and normalized probe signal (blue) and red arrow indicating the aberration for D2MCBL02. TD, Trilineage differentiation.

has been described as frequent during hiPSC culture [9], was confirmed by KaryoStat (Figure 2E). D2MCBL01B and D2MCBL03 displayed a normal karyotype using both KaryoStat and karyotype G-banding. CNV analysis of the WGS data did not reveal additional

abnormalities either. We were therefore confident that D2MCBL01B and D2MCBL03 had normal karyotypes.

WGS was performed on the pre-seed lots and WES was performed on the MCB and on higher passage D2P26L01B and

D2P26L03. Variant calling was performed against COSMIC and *de novo* variants not present in the donor PBMC, were scored. Variant calling was performed for information only and was not part of the release criteria. However, lines showing ACMG class 4 and class 5 variants were deemed unfit for release. *De novo* variant analysis of line D2P10L01 displayed 2 *de novo* variants ACMG class 3 (VUS). One was a heterozygous in-frame deletion in exon 37 of *PCM1* (rs749481887), and a heterozygous frameshift variant in exon 11 of *PAX8* introducing a premature stop codon (unknown variant). D2P10L03 displayed one heterozygous *de novo* variant (A to G) ACMG class 3 (VUS) in the 3' UTR region of *IGF2BP2* (rs544821602). These variants were observed in WGS analysis of the pre-seed lot P10 and remained present in the WES analysis of the MCB and passage 26. No additional variants were acquired up to passage 26. The observed *de novo* variants were verified using Sanger sequencing. This confirmed that the mutation rate in hiPSCs is low and that variants are probably introduced in early steps during- or after reprogramming. In both lines, *de novo* CNVs were absent. The combined results of MCB QC testing culminated in QP release of 2 MCB from D2 (D2MCBL01B, and D2MCBL04).

Differentiation to prospective clinical products

To test the capacity for differentiation to potential clinical products, hiPSC lines D2MCBL01B and D2MCBL03 underwent directed differentiation into various lineages. SC-islets generated from both lines ($n = 3$ for each line) generated on average $50.6 \pm 12.0\%$ SC β cells (C-peptide-expressing cells), $11.0 \pm 7.0\%$ SC α cells (glucagon-expressing cells) and $8.1 \pm 6.8\%$ polyhormonal cells (positive for both C-peptide and glucagon), validated by ICC (Figure 3A). Also, dithizone was used to selectively stain the SC-islet clusters red. Overall, SC islets produced from both lines exhibit a cytoarchitecture and composition that is similar to previously published SC islets and primary donor islets [37,38].

Kidney organoid formation was initiated using 15 000 or 25 000 hiPSCs/cm² ($n = 3$ for both conditions). High-quality kidney organoids were produced using both lines. D2MCBL01B performed slightly better with high-quality organoids for five of six differentiations compared with four of six good-quality organoids for line D2MCBL03. The presence of appropriate structures was confirmed with ICC using kidney-specific markers showing appropriately segmenting nephron structures with glomeruli (NPHS1+, NPHS2+) surrounded by endothelial cells (CD31+), proximal tubule (CUBN+, LTL+) and distal tubule (ECAD+) (Figure 3B) consistent with previous publications [18,19].

Cardiomyocyte differentiation was initiated using 20 000 or 25 000 hiPSCs/cm² ($n = 3$ for both conditions). Cardiomyocytes with spontaneously contracting regions were obtained around day 8 of differentiation (not shown). On average $55.0 \pm 8.8\%$ (D2MCBL01B) and $67.0 \pm 8.7\%$ (D2MCBL03) of cells expressed the pan-cardiomyocyte marker cardiac troponin T on day 17 of differentiation, confirmed by ICC staining for the sarcomere markers ACTN2 and TNNT2 (Figure 3C) [20].

Cells with basal keratinocyte characteristics were produced from both hiPSC lines, as defined by the expression of epidermal basal markers $\alpha 6$ - and $\beta 4$ -integrin subunits as well as p63 and keratin 14 [15]. Flow cytometry analysis showed similar yields with $70.7 \pm 14.4\%$ and $77.5 \pm 11.5\%$ of the cells being $\alpha 6$ - and $\beta 4$ -integrin double positive on day 20 ($n = 5$) (Figure 3D); furthermore, for D2MCBL01B and D2MCBL03, $78.1 \pm 18.3\%$ and $81.9 \pm 18.4\%$ of the cells expressed p63 and $83.4 \pm 16.9\%$ and $87.0 \pm 7.5\%$ of the cells expressed keratin 14 after the first passage ($n = 3$, not shown), respectively.

Neural lineage cells generated from D2MCBL01B ($n = 3$) and D2MCBL03 ($n = 1$) showed MGE progenitors gene expression profile

at day 16 of differentiation (NKX2.1+, ISL1+, FOXG1+, LHX6+ and PAX6-) and GABAergic interneurons profile at day 42 (NKX2.1+, SST+, GAD67+, LHX6+ and PVALB-). Neural lineage differentiation was confirmed by ICC with markers for MGE progenitors (NKX2.1+, ISL1+, PAX6-) and GABAergic interneurons (GABA+, SST+, MAP2+) for both lines (Figure 3E) [39,40]. D2MCBL03 MGE day 16 progenitors show low expression of ISL1, both at mRNA and protein level. Further analysis on day 42 showed maturation to SST+ GABAergic interneurons on both cell lines, however more replicates are needed to confirm the correct cell patterning.

Mature inner ear organoids could be produced using both lines. After optimizing the BMP4 concentration to induce otic differentiation, mature inner ear organoids were obtained containing hair cells embedded in epithelial vesicles with invading neuronal projections as confirmed by ICC at day 75 of culture. ICC confirmed the presence of these structures, MYO7A⁺ for hair cells and TUBB3⁺ for neurons, in both D2MCBL01B and D2MCBL03 (Figure 3F). The obtained results are congruent with previously reported data [22,23].

Structures that recapitulate aspects of somitogenesis, including the periodic appearance of somites, were also generated for both hiPSC lines. These somite-like structures undergo epithelialization, characterized by organization of actin and *uncx4.1* expression [25]. There were no statistical differences in length ($P = 0.45$), width ($P = 0.68$) and length-to-width ratio ($P = 0.57$) between somitoids generated with D2MCBL01B or D2MCBL03. Somitoids were classified into three categories: non-elongated somitoids (D2MCBL01B: 13.33%; D2MCBL03: 11.30%), ovoid somitoids (D2MCBL01B: 57.14%; D2MCBL03: 51.30%) and elongated somitoids (D2MCBL01B: 29.52%; D2MCBL03: 37.39%) (Figure 3G). Actin staining with phalloidin demonstrated epithelialization within the somites, and the marker for somite maturation UNCX4.1 was present in the most anterior somites but absent in the posterior somites (Figure 3G).

Successful differentiation was achieved for both GMP-released hiPSC lines into somitoids and six potential clinical products from all three germ layers: SC-islets (endoderm), kidney organoids and cardiomyocytes (mesoderm), and keratinocytes, GABAergic interneurons, and inner ear organoids (ectoderm).

Conclusions

We successfully developed and validated a GMP-compliant production platform for in-house hiPSCs production. This resulted in five pre-seed lots that passed all QC-testing and two MCBs that were QP-released and can be used as starting products for allogeneic clinical products. Furthermore, the platform as such will allow the production of patient-specific hiPSCs and their derivative cell therapy products for autologous therapies when the time arises. The establishment of this validated platform represents a critical milestone in the implementation of hiPSC-based regenerative medicine within our own university hospital and likely that of others. Moreover, the potential of the GMP-released MCBs to differentiate into putative clinical products highlights their suitability for therapeutic applications.

It is important to acknowledge that the methodology and materials we used here fall partly under patents of third parties and therefore the released products would have licensing restrictions when used. It will be up to users to negotiate licenses as necessary should they request use of these lines. Furthermore, clinical products from allogeneic hiPSCs would require immune suppression to counteract transplant rejection. In some cases, the immunogenicity of the organ for transplantation (like the eye and brain) may only require minimal or short periods of immune suppression. However, for highly immunogenic tissues (like the kidney and beta cells), immune evasive hiPSCs lines would be an alternative for off-the-shelf allogeneic cell therapies.

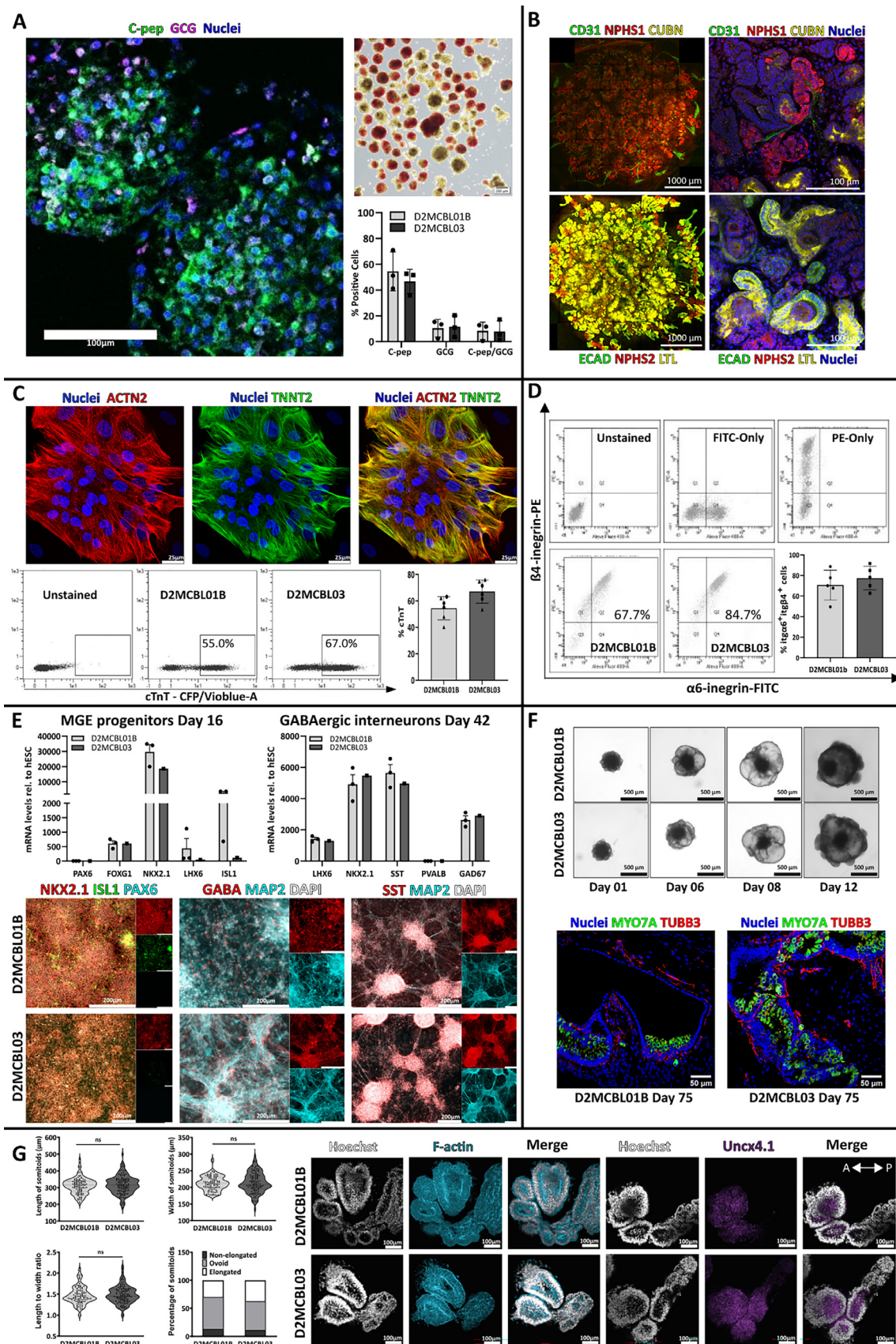


Figure 3. hiPSCs differentiation into putative cell products. (A) SC-islets: ICC (scale bars, 100 μ m) and dithizone (DTZ) staining (Scale bars, 200 μ m) in stage-7 SC-islet clusters from D2MCL01B. Percentage of C-peptide and glucagon positive cells (n = 3). (B) Kidney organoid: ICC of kidney organoids from D2MCL01B: endothelial cells (CD31, green), podocytes (NPHS1, red; NPHS2, red), proximal tubules (CUBN, yellow), LTL, yellow) and distal tubules (ECAD, green). (C) Cardiomyocytes: ICC of cardiomyocytes from D2MCL03: sarcomere markers ACTN2 (red) and TNNT2 (green). FACS analysis and yield of cTnT positive cells (n = 3 for each density). Circles and triangles seeding 20,000 or 25,000 hiPSCs/cm² respectively. (D) Keratinocytes: Representative FACS for $\alpha 6$ - and $\alpha 4$ - integrin subunits and yield of 5 independent differentiations. (E) Neural lineage: Relative gene expression at day 16

Declaration of Competing Interest

The authors have no commercial, proprietary or financial interest in the products or companies described in this article.

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Author Contributions

Conception and design of the study: BW, PM and TR. Acquisition of data: JN, ES, NF, CvdB, FC, BR, EG, KR, HL, WvdV, AK, AS, MY and PD. Analysis and interpretation of data: JN, BW, ES, PM, IW, CF and CA. Drafting or revising the manuscript: JN, BW, PM, CF, TR and CM. All authors have approved the final article.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at [doi:10.1016/j.jcyt.2024.02.021](https://doi.org/10.1016/j.jcyt.2024.02.021).

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and day 42 of neural progenitors and mature neuron markers (D2MCBL01B, n = 3; D2MCBL03, n = 1). ICC of MGE progenitors: NKX2-1 (red), ISL1 (green), PAX6 (cyan) and DAPI (gray). ICC of GABAergic interneurons: GABA (red), SST (red), MAP2 (cyan) and DAPI (gray) (Scale bar: 200 μ m). (F) Inner ear organoids: Bright-field images of the early time points of inner ear organoid differentiation. ICC images of day 75 aggregates: hair cells (MYO7A+, green) and neurons (TUBB3+, red) (n = 8). (G) Somatoids: 4 days after aggregation: length, width, and length-to-width ratios D2MCBL01B (n = 105) and D2MCBL03 (n = 115) and percentage of aggregates that formed somatoids. Somatoids 6 days after aggregation: Phalloidin (blue) or uncx4.1 (purple) Hoechst nuclear staining (white) (Scale bar: 100 μ m). cTnT, cardiac troponin T.